Synthesis of Functionalized Cyclohexadiene-*trans*-Diols with Recombinant Cells of *Escherichia coli***

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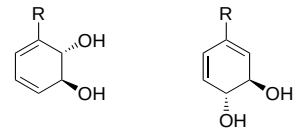
Functionalized cyclohexadiene-*cis*-diols (*cis*-CHD), that are produced by whole-cell bioconversion, have been demonstrated to be valuable precursors in the chemistry of natural products and pharmaceutics.[1] Using *cis*-CHD as a starting material, syntheses have been established, for example, for conduritol,[2] zeylena,[3] and *ent*-morphinan.[4]

In contrast, the corresponding cyclohexadienediols with *trans*-configured glycol unit (*trans*-CHD) have not been established as chiral synthetic building blocks yet, mainly due to the poor availability of these substances. On the one hand, *trans*-configured diols cannot be prepared by the dioxygenase-catalyzed dihydroxylation of arenes, whereas epoxidation with monooxygenases and subsequent nucleophilic opening usually gives racemic products. On the other hand, all chemical routes to *trans*-CHD are characterized by low enantioselectivity or tedious multistep syntheses.[5, 6] Attempts by several research groups to prepare *trans*-CHD directly from *cis*-CHD have similarly been characterized by low overall yield.[3, 7]

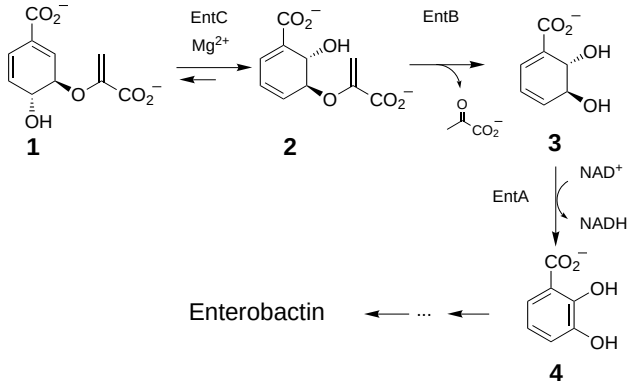


2,3-*trans*-CHD 3,4-*trans*-CHD

Nevertheless, it was shown that *trans*-CHD and especially derivatives of *trans*-2,3-dihydroxy-2,3-dihydrobenzoic acid (2,3-*trans*-CHD) are ideal precursors for the synthesis of aminocarbasugars (e.g. valienamine)[5b] and natural cyclohexanebisepoxides (e.g. crotepoxide).[8]

trans-CHDs also appear in nature as secondary metabolites of the shikimate pathway in bacteria, plants, and fungi, and therefore should, in principle, be accessible through metabolic engineering. Using this technique Frost et al., for instance, produced shikimic acid and quinic acid in concentrations up to 27 g L^{-1} and 13 g L^{-1}, respectively, by fermentation of recombinant strains of *Escherichia coli*.[9] Microbial production of two *trans*-CHDs (dihydroxydihydrobenzoic acid isomers) in concentrations up to 200 mg L^{-1} has been achieved by Leistner and co-workers with strains of *Klebsiella pneumoniae* (potentially human-pathogenic).[10] Therefore it is evident that *trans*-CHD could be simply and efficiently prepared in a biotechnological process that makes use of the shikimate pathway. Herein we describe the first direct access to (2*S*,3*S*)-dihydroxy-2,3-dihydrobenzoic acid (**3**) starting from glucose and other renewable carbon sources with metabolically deregulated, recombinant strains of *E. coli*.

Compound **3** is an intermediate on the pathway of the iron chelator enterobactin and is produced from chorismate **1** via isochorismate **2** in two catalytic steps (Scheme 1).[11] Enzymes involved in this process are isochorismate synthase (encoded by gene *entC*) and isochorismatase (encoded by *entB*). Aromatization to 2,3-dihydroxybenzoic acid (**4**) is catalyzed by 2,3-dihydroxybenzoate synthase (encoded by *entA*, Scheme 1). Intracellular overproduction of **3** can therefore be enhanced by two factors: 1.) by increase of the metabolic flux towards the target product, for example, by amplification of the EntB/EntC activities,[10] and 2.) by prevention of degradation of the target molecule, for example, by decrease of the EntA activity.



Scheme 1. Metabolites of the enterobactin pathway are chorismate **1**, isochorismate **2**, (2*S*,3*S*)-dihydroxy-2,3-dihydrobenzoic acid (**3**), and 2,3-dihydroxybenzoate (**4**). The participating enzymes are isochorismate synthase (EntC), isochorismatase (EntB), and 2,3-dihydroxybenzoate synthase EntA.

To show that **3** can be overproduced and excreted into culture medium, we have transformed *E. coli entA*$^-$ mutants (AN193, H1882) with plasmids containing *entB*, *entC*, or *entB*/*entC*. In addition, strains without *entA* defect (DH5α, W3110) were transformed analogously (Table 1).

Enhanced expression of the particular genes could be monitored by identification of the respective proteins on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using crude cell extracts of the recombinant strains. An increase of the corresponding enzyme activities could be clearly verified by using HPLC to monitor the degradation of chorismate **1** to **2** and **3**.

Shaking-flask fermentations proved that derivatives of the *entA*$^-$ mutants H1882 and AN193 with plasmid-borne genes *entB* **and** *entC* excreted **3** with a maximum production rate of 27 mg h^{-1} per g (dry cell mass). Strains DH5α and W3110 (with wild-type EntA activity and EntB/EntC overproduc-

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Table 1. *E. coli* K-12 parental strains in use.

Strain	Relevant genotype	*entA*	Ref.
DH5α	F$^-$ *endA1 hsdR17*($r_k^- m_k^+$) *recA1 supE44 thi-1* Δ(*lacZYA-argF*)*U169* Φ80*lacZ*ΔM15	+	[12]
W3110	F$^-$ λ IN(*rrnD-rrnE*) prototroph	+	[13]
AN193	*trpE38 leuB6 proC14 lacY1 fhuA23 rpsL109*(Str^R) λ^- *entA403*	–	[14]
H1882	*araD139* Δ(*argF-lac*)*169* λ^- *flhD5301 rpsL150*(Str^R) Δ(*fepA-ent*)	–	[15]

tion) also showed excretion of **3**; however, the excretion rate was only about 9 mg h^{-1} per g (dry cell mass), hence significantly lower than in strains with *entA* mutations (Table 2). Parent strains or strains which overproduced either EntB or EntC, showed no excretion of **3** or of other intermediates in the pathway of enterobactin (**1**, **2**, or **4**) into the fermentation broth.

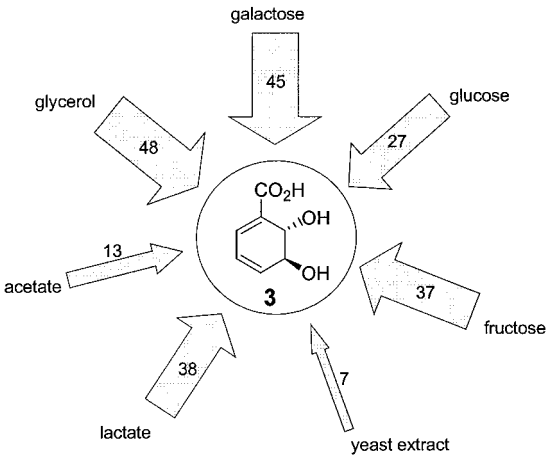
Table 2. Extent of excretion of **3** in [mg h^{-1} per g (dry cell mass)] depending on the overexpression of genes encoding for EntB or EntC, respectively. No excretion of **1**, **2**, or **4** was observed. Limit of detection was $\leq$ 1 mg L^{-1}.

Used strain (chromosomal specifity)	/pJF*entB* EntB	/pJF*entC* EntC	/pJF*entBC* EntB/EntC
DH5α, W3110 (*entA*$^+$)	n.q.[a]	n.q.[a]	9
AN193, H1882 (*entA*$^-$)	n.q.[a]	n.q.[a]	27

[a] n.q. = not quantifiable (below limit of detection).

To avoid inhibitory or repressive influences of complex components of the medium on product formation, a minimal salt medium has been developed consisting of mineral salts solution, buffer, and a defined carbon source. Best growth and production rates have been obtained by using a previously published mineral salts medium[16] with increased concentration of ammonium chloride. In addition, the medium was supplemented with 2,3-dihydroxybenzoate (due to the *entA* deficiency), and growth supplements to satisfy the auxotrophies for thiamine, leucine, proline, and tryptophan were added in all experiments.

Apart from glucose, **3** can be readily produced from other C sources, such as galactose, fructose, glycerol, acetate, or lactate (Scheme 2). The two best C sources (glycerol, galactose) resulted in even better excretion performances (up to 48 mg h^{-1} per g (dry cell mass)).



Scheme 2. Microbial production of **3** is possible starting from various renewable carbon sources. The values given refer to the production rates [mg h^{-1} per g (dry cell mass)] obtained for cells of the *E. coli* strain AN193 with genes *entB*/*entC* in mineral salts medium with 10 g L^{-1} substrate.

With regard to the production of **3**, the strains with an *entA*$^-$ mutation showed long-term stability as well as high production rates—basic requirements for high space – time yields in continuously working production processes. By using a pH-controlled stirred tank reactor and applying optimized conditions (pH 6.8, 37 °C, glucose-feeding in a fed-batch mode), the catalytic reaction could be maintained during a process time of more than 40 h. During this time 92 g of **3** were produced from 690 g of glucose monohydrate in a 20 L cultivation experiment at a cell density of 12 g L^{-1} (dry cell mass), correlating with a molar yield of 17 %.[17] Here again **3** was the major product with no detectable impurities of **1**, **2**, or **4.** Therefore any further purification with laborious separation techniques becomes obsolete.

A simple and quantitative separation from the fermentation salts can be achieved by multiple extraction of the acidified (pH 3) permeate of the cell suspension, using short-chain alcohols, (e.g. 1-butanol; extraction coefficient 30 %). After purification by column chromatography **3** is isolated in 73 % yield as a yellow solid in highly pure form. Alternatively, the product can be isolated with similar purity and in 75 % yield by using ion exchange chromatography.[18] At room temperature and moderate pH ($3 \leq$ pH ≤ 11) **3** is stable over a period of weeks.

To demonstrate that quite similar follow-up chemistry to that observed with *cis*-CHD is also possible for the substituted *trans*-CHD, we exemplarily worked out highly regio- and stereoselective syntheses starting from **3** towards pharmacologically interesting target molecules.[19]

In summary, we have demonstrated that (2*S*,3*S*)-dihydroxy-2,3-dihydrobenzoic acid (**3**) can be prepared directly starting from glucose and other renewable carbon sources, using metabolically deregulated, recombinant *E. coli* strains. With these strains at hand, which are both nonpathogenic and are classified as S1 organisms according to German recombinant DNA regulations, it is now possible for the first time to produce **3** on a multigram scale in a cost-efficient manner. It is exceptionally advantageous that the product is free of metabolic impurities, so that it can be isolated easily. We are presently also working on the enantioselective access to derivatives of 3,4-*trans*-CHD through metabolic deregulation in recombinant microorganisms.

Experimental Section

Genes *entB* and *entC* were amplified in vitro by polymerase chain reactions (PCR) using chromosomal DNA of *E. coli* K-12 wild-type strain W3110. The oligonucleotides used were 5′-TATGGATCCACGCGCATCAGCCTGAA-3′ and 5′-GGGCTGCAGACATTTTTACCGCTG-3′ for *entB* and 5′-GGCGAGCTCATTATTAAAGCCTTT-3′ and 5′-TGCGGATCCTCGCTCCTTAATGC-3′ for *entC*. The resulting amplification products with

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engineered restriction sites were inserted into the cloning plasmid pJF119EH1[20] both separately or in tandem. Cloning sites were *Bam*HI and *Pst*I for *entB*, and *Sac*I and *Bam*HI for *entC*, respectively. The *E. coli* strains W3110, DH5α, AN193, and H1882 were transformed with each of the resulting three plasmids.

Fermentations for product formation analysis were performed in a mineral salts medium (100 mL; pH 7 maintained by addition of 1N NaOH)[10] in 1 L shake flasks. Cells from a 100 mL preculture in Luria–Bertani medium[21] (containing 100 mg L^{-1} ampicillin, 100 μM isopropyl β-D-thiogalactopyranoside (IPTG) at an optical density ($\lambda = 600$ nm) of 2) were harvested in the mid-logarithmic phase and were resuspended in mineral salts medium (containing 100 mg L^{-1} ampicillin, 100 μM IPTG) and were incubated at 37 °C and 150 rpm. Product formation was monitored during a period of 10 h by HPLC analysis (maximal excretion was after ca. 4 h).

Mineral salts medium for optimized production and growth (derived from Pan et al.[16]) contained per liter: KH_2PO_4 (13 g), K_2HPO_4 (10 g), $NaH_2PO_4 \cdot 2H_2O$ (6 g), $(NH_4)_2SO_4$ (2 g), $MgSO_4 \cdot 7H_2O$ (3 g), NH_4Cl (5 g), $FeSO_4 \cdot 7H_2O$ (40 mg), $CaCl_2 \cdot 2H_2O$ (40 mg), $MnSO_4 \cdot 2H_2O$ (10 mg), $ZnSO_4 \cdot 7H_2O$ (2 mg), $AlCl_3 \cdot 6H_2O$ (10 mg), $CoCl_2 \cdot 6H_2O$ (4 mg), $Na_2MoO_4 \cdot 2H_2O$ (2 mg), $CuCl_2 \cdot 2H_2O$ (1 mg), H_3BO_3 (0.5 mg), leucine (200 mg), proline (200 mg), adenine (200 mg), thiamine (20 mg), tryptophan (200 mg), 2,3-dihydroxybenzoic acid (20 mg), and ampicillin (100 mg).

The cultivation in a 30 L stirred tank bioreactor (Chemap, Switzerland, 20 L working volume) was performed at pH 6.8 (controlled) and 37 °C with optimized medium. 1 L preculture from complex medium served as inoculum. The initial concentration of glucose was 30 g L^{-1}. The carbon source was added in portions so that the concentration was maintained in the range of 5–10 g L^{-1}. Induction by addition of 100 μM (final concentration) IPTG was at a cell concentration of 5 g L^{-1} (dry cell mass). The aeration rate was regulated to 25 L min^{-1}(air).

The crude product was purified by column chromatography on silica gel 60 using ethyl acetate/MeOH (5/1) as eluent. Physical data of **3** (yellow solid): $[\alpha]_D^{20} = 3.8$ ($c = 0.6$ in ethanol); ^{1}H NMR (300 MHz, [D$_4$]MeOH, 23 °C): $\delta =$ 4.10 (d, ^3J(H,H) = 2.5 Hz, 1H; C*H*OH), 4.50 (d, ^3J(H,H) = 2.5 Hz, 1H; C*H*OH), 6.20 (m, 2H; C=C*H*), 7.06 (dd, ^3J(H,H) = 3.3 Hz, ^4J(H,H) = 3.2 Hz, 1H; C=C*H*); ^{13}C NMR (75 MHz, [D$_4$]MeOH, 23 °C): $\delta = 68.8$ (CH), 70.4 (CH), 125.1 (CH), 130.8 (C_{quart}), 134.3 (CH), 134.4 (CH), 170.2 (COOH); IR(KBr): $\tilde{\nu} = 1699$ cm^{-1} (C=O), 1644 (C=C), 1586 (C=C), 1258, 1075, 1008; UV/Vis (H_2O): $\lambda_{max}(\varepsilon) = 279$ nm (4900); EI-MS (70 eV): *m*/*z* (%): 156 (6) [M^+], 138 (100) [$M^+ - H_2O$], 110 (63) [$M^+ - 2H_2O$], 93 (10), 82 (29), 65 (13) [$C_5H_5^+$]; HR-MS: calcd for $C_7H_8O_4$: 156.0423; found: 156.0424.

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On the Aromatic Character of Electrocyclic and Pseudopericyclic Reactions: Thermal Cyclization of (2*Z*)-Hexa-2,4-5-trienals and Their Schiff Bases**

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Recent research on the magnetic characterization of aromaticity[1] has, over the last few years, promoted the reexamination of the aromatic character of pericyclic reactions.[2] This idea stems from the pioneering work of Evans,[3a] Dewar,[3b] and Zimmerman[3c] and has been refined by Herges, Jiao, and Schleyer.[2] Recently, we reported[4] that the concept of in-plane (σ) aromaticity can be extended to several thermal pericyclic reactions. Within this context, we herein report several ring closures involving (2*Z*)-hexa-2,4,5-trienals and their corresponding imines (Scheme 1). These compounds are

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